AD_____

Award Number: DAMD17-00-1-0074

TITLE: p16 Axis in Androgen-Dependent Proliferation of Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Liang Zhu, M.D., Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx, NY 10461

REPORT DATE: April 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Final(1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE

P16 Axis in Androgen-Dependent Proliferation of Prostate Cancer Cells

5. FUNDING NUMBERS

DAMD17-00-1-0074

6. AUTHOR(S)

Liang Zhu, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Albert Einstein College of Medicine
Bronx, NY 10461

E-Mail: lizhu@aecom.yu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to understand the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressors (p16Ink4a and Rb), cyclin D-dependent kinases, and transcription factor E2F. We hypothesized that functions of the p16 axis can influence androgen-dependence of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in human androgen-dependent prostate cancer cells (LNCaP) and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells (DU-145). We have now determined that the androgen receptor itself plays an important role in androgen-dependent proliferation of LNCaP cells. Furthermore, when the expression of the androgen receptor is restored together with the tumor suppressor Rb, a cell death pathway can be activated in DU-145 cells. These findings provide new understanding of the underlying mechanisms of prostate cancer proliferation in response to androgen and may provide potential strategy for prostate cancer gene therapy.

14. SUBJECT TERMS

Androgen dependence, cell cycle regulation, tumor suppressors, Androgen receptor, inducible expression cell lines, cell death

15. NUMBER OF PAGES

17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The subject of this proposal is the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressers (p16Ink4a and Rb), an important component of the cell cycle machinery (cyclin D-dependent kinases), and transcription factor E2F (regulators of gene expression). We hypothesized that functions of the p16 axis can influence androgen regulation of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in androgen-dependent prostate cancer cells and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells. We expected that this study will reveal connections between androgen regulation of prostate cancer cells and functions of a central cell growth control pathway. A major outcome of this study that we are able to present in a published article is that restoration of Rb and AR expression in Rb and AR deficient prostate cancer cells can induce apoptosis. Importantly, this apoptosis is entirely dependent on the restoration of both Rb and the AR. We discovered that Rb can stimulate the transactivation activity of the AR and this activity induces mitochondria damage, caspase cleavage, and cell death. Our studies of androgen-dependent prostate cancer cells led to an unexpected finding that the expression levels of the AR itself is responsible for the proliferation arrest upon androgen withdrawal. Our preliminary results suggest that Skp2, a component of the SCFRoc1-Skp2 ubiquitin E3 ligase, might be a downstream effector of the AR in mediating androgen-independent proliferation. Studies are ongoing to determine the role of Skp2 in androgen responsiveness of prostate cancer cells.

Body

The two approved aims in this project are discussed separately below.

Aim 1. To determine whether deregulated expression of positive-acting cell cycle regulators can abrogate the dependence of LNCaP cells on steroid hormones in culture.

The key step in this Aim as originally designed is to use the tetracycline–controlled inducible expression system to establish LNCaP derivative cell lines that allow inducible expression of various cell cycle regulators. The tetracycline-controlled inducible expression system is a two-step process. The first step is to establish a derivative cell line that expresses the tetracycline-controlled transactivator (tTA). From this tTA cell line, a second derivative line is then established that contains the gene under study under the control of the tTA-controlled promoter. We experienced significant difficulties from the poor ability of LNCaP cells to form single colonies under drug selection (a necessary step in establishing clonal cell lines after transfection) as well as to expand from such single colonies in culture. Using a modified retrovirus-mediated gene transfer method, we succeeded in generating LNCaP tTA cell lines. All attempts to establish the secondary line with inducible expression of cell cycle regulators however have not been successful. More recently, we have obtained good results using lentivirus-mediated gene transfer to establish LNCaP derivative cell lines (see below).

In characterizing functional status of cell cycle regulators in LNCaP cells in response to androgen, we found that protein levels of the AR decreased dramatically when LNCaP cells were switched from media contain regular fetal bovine serum (FBS) to media containing charcoal dextran treated serum (CDT) (Figure 1A, lanes 1 and 2). AR protein levels recovered and cell proliferation resumed when the cells were subsequently incubated with the CDT media supplemented with DHT at 1 nM (data not shown). These findings suggested to us that cell cycle arrest of LNCaP cells in response to androgen deprivation could be the result of the lack of the androgen receptor, which would complicate the studies of the roles of cell cycle regulators in any androgen-dependent response. Therefore, we decided to investigate whether prevention of AR down regulation would have any effects on the androgen response of LNCaP cells. To achieve this, we introduced the AR gene under control of the CMV promoter through stable infection with a CMV-AR containing

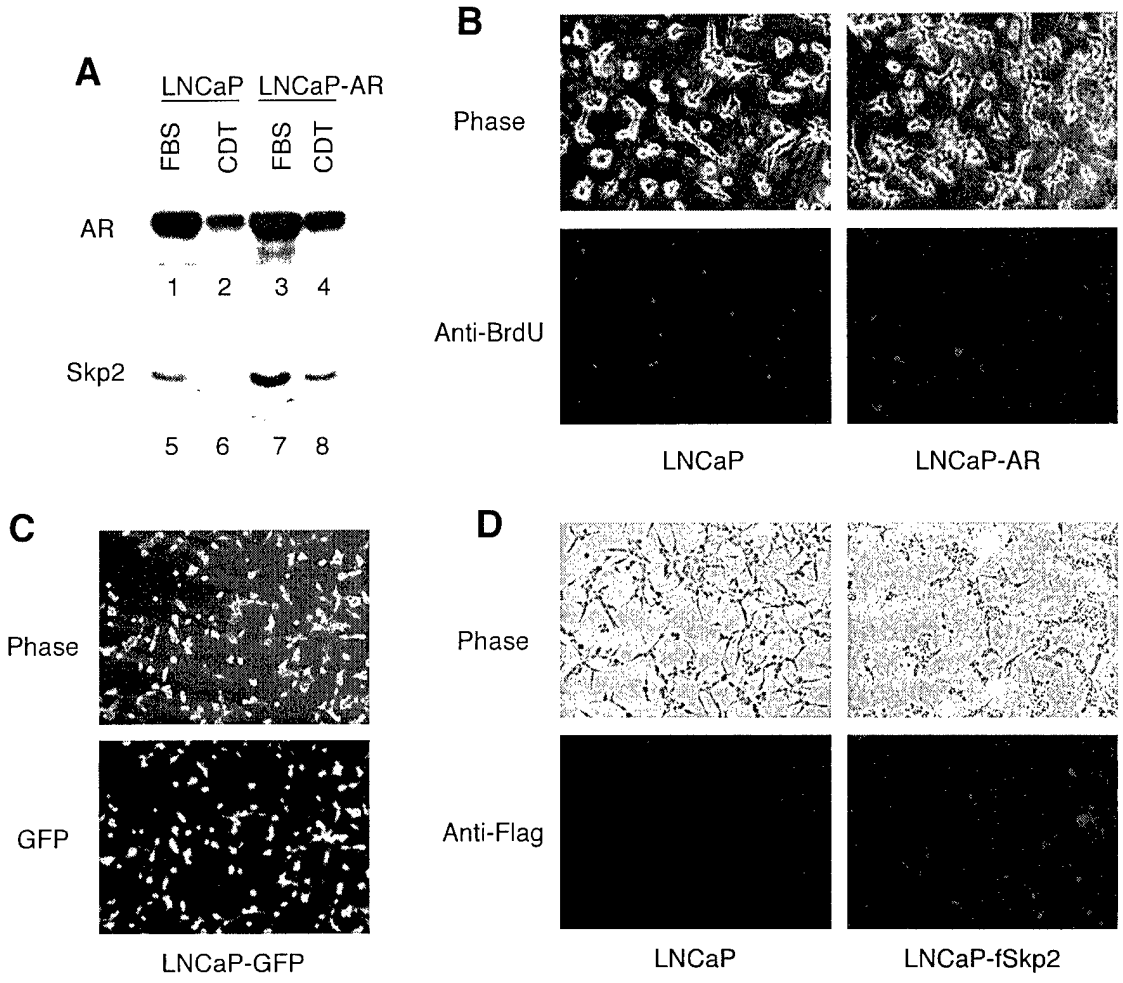

Figure 1. Regulation and function of the AR and Skp2 in LNCaP cells in response to androgen withdrawal. A. LNCaP and LNCaP-AR cells were cultured in FBS or CDT containing media for three days pair-wise. Protein levels of the AR and Skp2 were determined with Western blotting with the indicated antibodies. B. LNCaP and LNCaP-AR cells were cultured as in panel A except BrdU was added to the media for the last six hours. Cells were fixed and stained for BrdU positive cells. C. LNCaP cells were infected with a CMV-GFP lentivirus for three days. D. LNCaP cells were infected with a CMV-fSkp2 lentivirus. Cells were fixed and stained with an anti-flag antibody.

retrovirus to establish LNCaP-AR cells. As shown in Figure 1A lanes 3 and 4, LNCaP-AR cells contained about 2-3 fold more AR in regular (FBS containing) media compared with the parental LNCaP cells in the same condition (compare lanes 1 and 3). Although reduced, protein levels of the AR in LNCaP-AR cells clearly remained significant after androgen withdrawal (compare lanes 2 and 4). LNCaP-AR cells are significantly more proliferative (as determined by BrdU incorporation) in androgen-depleted media than the parental LNCaP cells (Figure 3B), suggesting that the lower AR levels in response to androgen withdrawal is at least partially responsible for the proliferation arrest of LNCaP cells after androgen withdrawal. While this work was in progress,

Chen et al published their working with nearly identical results (Chen et al., 2004). Together, these results reveal that androgen-dependent proliferation of prostate cancer cells, at least with the LNCaP cell model, could be explained by the regulation of AR signaling itself.

We next decided to further our study by investigating the events downstream of AR signaling that are responsible for promoting cell proliferation independent of androgen. One example of such study is shown in Figure 1A. Protein levels of Skp2 was significantly reduced upon androgen withdrawal (lanes 5 and 6). When the AR level was kept high in LNCaP-AR cells after androgen withdrawal, Skp2 level also remained high (compare lanes 6 and 8). Skp2 recruits, among others, the cyclin-dependent kinase inhibitor p27 for ubiquitination and degradation and has oncogenic activity. Based on our finding, we hypothesized that the repression of Skp2 levels might be the mechanism for proliferation inhibition of LNCaP cells after androgen withdrawal; and the maintenance of Skp2 levels might be responsible for the increased proliferation seen in LNCaP-AR cell lines after androgen withdrawal (Figure 1B). To test this hypothesis, we established LNCaP derivative cells with constitutive high expression of Skp2.

In this experiment, we established a different method to generate derivative LNCaP cells that avoided the necessity of clonal selection and expansion. We have established protocols to produce high titer lentivirus stocks that can infect more than 90% of the LNCaP cells in culture. This is demonstrated with a CMV-GFP lentivirus shown in Figure 1C. Lentivirus infection leads to stable integration of the viral genome and therefore allows the establishment of stable derivative cells without the need of drug selection.

With this method, we have established LNCaP derivative cells with a lentivirus vector expressing flag tagged Skp2 (fSkp2). As shown in Figure 1D, nearly 100% of the LNCaP cells stained positive with the anti-Flag antibody. Expression of Skp2 was stable in subsequent culture (data not shown). Experiments to determine whether higher levels of Skp2 expression could lead to androgen-independent proliferation of LNCaP cells are currently ongoing. Results from this series of experiments will significantly improve our knowledge about underlying mechanisms of androgen-dependent and –independent proliferation of prostate cancer cells.

Aim 2. To determine whether correction of p16 axis function and androgen receptor expression can restore androgen dependence in DU-145 cells.

The widely used DU-145 prostate cancer cell model lacks wild type Rb and AR expression and is androgen-independent. The first step in our experimental design is to restore the expression of Rb and the AR. The major outcome from studies in this Aim is that re-expression of Rb together with the AR in DU-145 cells resulted in an apoptotic activity, acting through the mitochondria damage-initiated caspase activation pathway, that was not present when RB, or the AR, was re-expressed alone. The ability of RB+AR to induce mitochondria damage was dependent on the pro-apoptotic proteins Bax and Bak and could be blocked by the anti-apoptotic protein Bcl-xl. Co-expressed AR did not detectably change Rb's regulation of E2F and cell cycle progression in culture. On the other hand, co-expressed Rb could activate AR's transactivation activity in an androgen depleted media. Although androgen induced greater AR transactivation activity in this condition it did not induce apoptosis in the absence of co-expressed Rb. Analysis of mutants of Rb and the AR indicated that intact pocket function of Rb and the AR's transactivation activity were required for Rb+AR induced apoptosis. These results provide direct functional data for an AR-dependent apoptosis-inducing activity of Rb, and highlight the importance of cell type-specific regulators in obtaining a more complete understanding of Rb. This work has been published (Wang et al., 2004), please see the attached reprint for details.

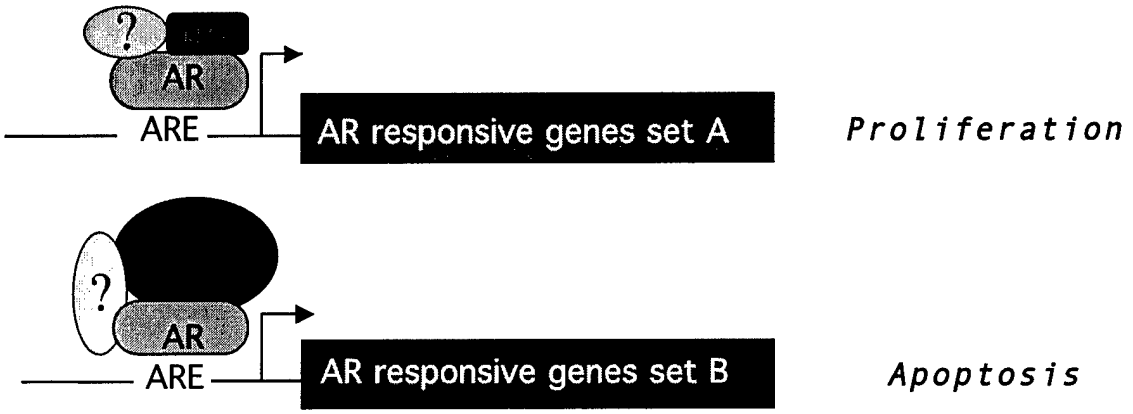

Figure 2. A working model for Rb+AR induced apoptosis. Based on our results, we propose the following model for the functional relationship between Rb and the AR. Rb interacts with the AR to activate its transaction activity. The Rb-mediated activation of the AR may lead to the expression of a set of AR responsive genes, whose products may function to induce apoptosis. Activation of AR by DHT in the absence of Rb may lead to the expression of a different set of genes, whose products promote the already-established role of the AR in prostate cell proliferation and differentiation. Other factors may also be involved in this differential regulation of the AR.

Based on this work, we hypothesized that Rb can interact with the AR to facilitate the expression of a set of genes that are different from those expressed by the androgen-stimulated AR (see Figure 2). We have initiated new experiments to identify genes that are activated by the AR in a Rb-dependent manner. In these experiments, we have used Affymetrix Human Genome U133 set containing 33,000 well substantiated human genes. We are comparing genes that are induced specifically by the induction of Rb with genes that are induced by androgen. We anticipate that results from this study will provide a clearer understanding of the mechanisms by which the AR and Rb induce prostate cancer cell death.

Key Research Accomplishments

- Through combined restoration of Rb and the AR in Rb and AR mutant prostate cancer DU-145, we identified a novel, apoptosis-inducing activity of Rb that is entirely dependent upon the transactivation activity of the AR.
- Through characterization of the androgen responses of LNCaP cells, we identified that the protein levels of the AR play an important role in cell cycle arrest after androgen withdrawal. A downstream effector of the AR signaling is likely the protein Skp2.

Reportable Outcomes

- Induction of Androgen Receptor-Dependent Apoptosis in Prostate Cancer Cells by the Retinoblastoma Protein
- The role of the Androgen Receptor signaling and Skp2 in androgen-dependent proliferation of prostate cancer cells.

Conclusions

Studies conducted under Aim 1 have uncovered a role of AR protein levels in androgen-dependent proliferation of LNCaP cells. This effect of the AR correlated with the regulation of protein levels of Skp2. LNCaP derivative cells with high Skp2 expression levels have been established to determine the role of Skp2 in androgen dependent proliferation of LNCaP cells.

Skp2 targets the kinase inhibitor p27 for ubiquitin-mediated degradation and has been found to have oncogenic activity and often overexpressed in human cancers. The determination of the role of Skp2 in androgen-dependent proliferation of prostate cancer cells will shed new light on this critical aspect of prostate biology.

Experiments proposed in Aim 2 of this grant provided new understanding of the roles of the tumor suppressor Rb and the AR in prostate cancer cells. One functional role of the tumor suppresser Rb in prostate cells may be to promote cell death in an AR-dependent manner. Prostate cancer cells may gain a survival advantage when Rb and/or the AR is mutated during the disease course. The requirement for both Rb and the AR for apoptosis should have implications in prostate cancer gene therapy.

Accomplishment in both Aims have led us to new studies (currently ongoing) to determine the molecular mechanisms of androgen dependent and independent proliferation of prostate cancer cells with close relevance to prostate cancer therapy.

References

Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004). Molecular determinants of resistance to antiandrogen therapy. Nat Med *10*, 33-39.

Wang, X., Deng, H., Basu, I., and Zhu, L. (2004). Induction of androgen receptor-dependent apoptosis in prostate cancer cells by the retinoblastoma protein. Cancer Res *64*, 1377-1385.

Bibliography of publications supported by this grant

Wang, X., Deng, H., Basu, I., and Zhu, L. (2004). Induction of androgen receptor-dependent apoptosis in prostate cancer cells by the retinoblastoma protein. Cancer Res *64*, 1377-1385.

Personnel (partially) supported by this grant

Liang Zhu, MD, PhD
Xingtao Wang, PhD
Indranil Basu, PhD

Appendix

Copies of original article: Wang, X., Deng, H., Basu, I., and Zhu, L. (2004). Induction of androgen receptor-dependent apoptosis in prostate cancer cells by the retinoblastoma protein. Cancer Res *64*, 1377-1385.

[CANCER RESEARCH 64, 1377–1385, February 15, 2004]

Induction of Androgen Receptor-Dependent Apoptosis in Prostate Cancer Cells by the Retinoblastoma Protein

Xintao Wang, Haiyun Deng, Indranil Basu, and Liang Zhu

Department of Developmental and Molecular Biology, The Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Bronx, New York

ABSTRACT

Re-expression of a tumor suppressor in tumor cells that lack it is an effective way to study its functional activities. However, because tumor cells contain multiple mutations, tumor suppressor functions that are dependent on (an)other regulators are unlikely to be identified by its re-expression alone if the other regulators are also mutated. In this study, we show that re-expression of retinoblastoma (RB) together with the androgen receptor (AR) in RB- and AR-deficient prostate cancer DU-145 cells resulted in an apoptotic activity, acting through the mitochondria damage-initiated caspase activation pathway, which was not present when RB, or the AR, was re-expressed alone. The ability of RB + AR to induce mitochondria damage was dependent on the proapoptotic proteins Bax and Bak and could be blocked by the antiapoptotic protein Bcl-x_L. Coexpressed AR did not detectably change RB's regulation of E2F and cell cycle progression in culture. On the other hand, coexpressed RB could activate the transactivation activity of the AR in an androgen-depleted media. Although androgen induced greater AR transactivation activity in this condition, it did not induce apoptosis in the absence of coexpressed RB. Analysis of mutants of RB and the AR indicated that intact pocket function of RB and the transactivation activity of the AR were required for RB + AR-induced apoptosis. These results provide direct functional data for an AR-dependent apoptosis-inducing activity of RB and highlight the importance of cell type-specific regulators in obtaining a more complete understanding of RB.

INTRODUCTION

The tumor suppressor retinoblastoma (RB) and the RB-E2F pathway play central roles in cell proliferation and tumorigenesis (see Ref. 1 for a recent review). RB functions as a transducer between the cell cycle engine and the cellular gene expression programs, most significantly the E2F-regulated gene expression program, to control cell proliferation at various cell cycle transition points, most importantly at the G_1-S transition. In addition to this general, non-cell type-specific role, it is generally believed that RB also has cell type-specific functions. This latter aspect of RB, although much less well understood, is clearly an integral part of RB function because RB mutations in cancer have cell type-specific patterns. RB mutations are primarily found in retinoblastoma, osteosarcoma, lung, prostate, breast, and bladder cancers (2).

A commonly used and very effective approach to revealing the functional activities of tumor suppressors is to re-express them in tumor cells that lack them because of mutations selected for during the tumorigenesis process. Re-expression of RB in RB-deficient tumor cells generally leads to repression of E2F and G_1 cell cycle arrest; and data from the re-expression experiments constitute a major part in the current model of RB function as mentioned above. A hallmark of tumorigenesis, however, is that cancer cells almost always contain multiple alterations (3). Based on this fact, re-expression of a tumor suppressor in the absence of other presumably important regulators (because they are also mutated in tumorigenesis) is unlikely to reveal all of the functional activities of this particular tumor suppressor (because certain functions of this tumor suppressor may depend on certain other regulators).

We have applied these considerations to the study of RB in prostate cancer cells. Combined results from many studies have demonstrated that the RB gene is mutated in about 20% of the prostate cancer samples at both early and late stages of this disease (4–7). The widely used prostate cancer cell model DU145 contains exon 21 deletion in its RB gene (4), which abolishes the pocket functions of RB (therefore considered as a null mutation). Re-expression of wild-type RB in DU145 cells did not have cell cycle effects in culture (4). The androgen receptor (AR) plays important roles in prostate cell proliferation, differentiation, and survival and is often mutated in prostate cancer, particularly in late stages (8–10). DU145 cells do not express the AR. It has been demonstrated previously that RB and the AR can physically interact and RB can stimulate AR-mediated transactivation (11, 12). In this study, we determined the effects of re-expressing RB and the AR separately and in combination in DU145 cells. This experimental approach revealed an AR-dependent apoptosis-inducing activity of RB.

MATERIALS AND METHODS

Cell Lines. Prostate cancer cell lines DU145 and LNCaP were purchased from American Type Culture Collection and cultured in DMEM media containing 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Charcoal dextran treated fetal bovine serum was purchased from Gemini, dihydrotestosterone (DHT) from Sigma, and the synthetic androgen R1881 from DuPont Merck Pharmaceutical Co. Androgen antagonist Casodex (bicalutamide) was a gift from AstraZeneca (Cheshire, United Kingdom). DU145 cells were transfected with pUHD-172neo (encoding the reverse tetracycline-regulated transactivator rtTA; provided by Dr. H. Bujard). G418-resistant clonal cell lines were established and screened for their ability to express a reporter gene under the control of the tetracycline-responsive promoter (pUHD10–3; provided by Dr. H. Bujard). These clones are named DU-rtTA. DU-rtTA cells were then stably transfected with pUHD10–3-RB (13) and pBabePuro to establish puromycin-resistant DU-RB cell lines. Induction of RB expression in DU-RB cell lines was achieved by treating these cells with doxycycline hydrochloride (Dox, Sigma) at 1 μg/ml. DU-RB cells were subsequently transfected with pCMV-ARzeo or pCMV-AR-KAzeo [the AR and AR-KA (mutation of K630 to A) cDNAs were provided by Dr. R. Pestell] to establish zeocin (0.1 μg/ml final concentration) resistant DU-RB-AR and DU-RB-KA cell lines, respectively. For analysis of RB mutant Δ22, identical methods were used to generate DU145 cell lines that inducibly express RBΔ22 and constitutively express the AR (DU-Δ22-AR).

Cell Proliferation and Apoptosis Assays. BrdUrd pulse labeling was performed with a final concentration of 50 nM for 4 h with cells cultured on glass cover slips in various conditions. Cells were fixed with cold ethanol, denatured with 2 N HCl, and stained with anti-BrdUrd (Calbiochem) and secondary goat antimouse IgG conjugated with FITC. BrdUrd-positive cells were identified under fluorescent microscope and the percentage of BrdUrd-positive cells in the whole population determined. Photograph of cultured cells under a phase-contrast microscope and the determination of cell numbers were conducted with standard procedures. To measure apoptosis with sub-G_1 fluo-

Received 8/5/03; revised 11/21/03; accepted 12/17/03.

Grant support: United States Army Prostate Cancer Research Program (DAMD17-00-1-0074).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Notes: X. Wang and H. Deng contributed equally to this work. L. Zhu is a scholar of the Leukemia and Lymphoma Society of America.

Requests for reprints: Liang Zhu, Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Room U-519, Bronx, New York 10461. Phone: (718) 430-3320; Fax: (718) 430-8975; E-mail: lizhu@aecom.yu.edu.

rescence-activated cell sorter analysis, induced cells (both floating and attached) were harvested, fixed in ethanol, stained with propidium iodide, and analyzed by a fluorescence-activated cell sorter Scan with CellQuest software. Green fluorescent protein-Annexin V staining was performed with a kit from Clonetech. To measure mitochondrial membrane potential, cells were incubated for 30 min in 40 nM $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide; Molecular Probes) before harvest. Total cells were then harvested, washed with PBS, and analyzed by fluorescence-activated cell sorter. Bcl-x_L-expressing adenovirus was provided by Dr. J. D. Molkentin. Virus stocks were prepared by the CsCl purification method, and the amounts of viruses needed to infect >80% of the cells were determined by immunofluorescence staining of Bcl-x_L. Cells were first infected with adenovirus for 24 h and then induced to express RB as usual. When apoptosis was induced by chemicals, cells were treated with okadaic acid at 20 nM or etopside at 50 μM for 24 h. To determine the functional status of cellular p53, cells were treated with actinomycin D at 20 nM for 24 h.

Immunoprecipitation and Western Blot Assays. Western blot analysis was conducted as described previously (14) except for the detection of caspases and poly(ADP-ribose) polymerase, for which we followed the protocol described previously (15). For immunoprecipitation experiments, cell extracts prepared from two 10-cm plates of cells (containing 1000 μg of total protein) were incubated with 2 μg of antibody on ice for 4 h, and immunocomplexes were precipitated with 25 μl of a mix of protein A and G beads in 4°C with rocking. The immunoprecipitates were washed three time with the lysis buffer (250 mM NaCl, 50 mM HEPES (pH 7.0), 5 mM EDTA, 0.1% NP40, 2 mM phenyl methylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 20 μg/ml trypsin inhibitor, 1 mM Benzamidine) before being analyzed by Western blotting. Antibodies against the AR (C-19 and 441), Bax (N-20), Bak (G-23), Bcl-2 (N-19), Bcl-x_L (M-125), cyclin E (C-19), cyclin A (H432), Cdk2 (M-2), Cdk4 (C-22), Cdc2 (17), Cdc25A (F-6), p27 (C19), p21 (F-5), and p53 (DO-1) were purchased from Santa Cruz Biotechnology; poly(ADP-ribose) polymerase (9542), caspase 3 (9661), caspase 9 (9502), and caspase 8 (9746) were obtained from Cell Signaling; and RB (clone G3–245) was purchased from BD PharMingen. Anti-E2F1 (KH-20) was provided by Dr. K. Helin.

Promoter Reporter Assays. DU145 cells were plated in 6-well plates at 5×10^5 per well in fetal bovine serum (FBS) media. One day after plating, the cells were transfected with LipofectAMINE Plus with 3 μg of total DNA. The E2F reporter E2F-TK-Luc and the AR reporter mouse mammary tumor virus-Luc were provided by Dr. R. Pestell. The expression vector for a full-length RB with all potential phosphorylable Thr/Ser changed to Ala was provided by Dr. J. W. Harper. At the end of a 6 h transfection period, cells were switched to charcoal dextran-treated serum (CDT) media. When needed, DHT at 1 nM final concentration and Dox at 1 μg/ml final concentration were added for the last 7 h. R1881 was also used to stimulate the AR, which yielded similar results as DHT (data not shown). Casodex at 20 μM was used as androgen antagonist. Cell lysates were prepared by directly adding lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*',*N*',*N*',*N*'-tetraacetic acid, 10% glycerol, and 0.2% Triton X-100] to the cells on ice. Luciferase activity was determined with luciferase assay systems from Promega Corp. following the manufacturer's protocol with an Autolumat LB 953 (EG&G Berthold). Luciferase activity was normalized for transfection efficiency with a cotransfected β-galactosidase reporter.

Small Interfering (si)RNA-Mediated Knockdown of Bax and Bak. DU-RB-AR cells were plated in 6-well plates and transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocol. siRNA against Bax and Bak were purchased from Dharmacon's SMARTpool selection; these Bax and Bak siRNA pools are tested and proven to knockdown at lease by 75% of Bax and Bak, respectively. A negative control pool was included in all siRNA experiments. Forty-eight h after transfection, cells were split into fresh media with and without Dox for another 36 h before analysis.

RESULTS

Re-expression of RB and the AR and Their Physical Interaction in Prostate Cancer DU145 Cells. We used prostate cancer cell model DU145, which lacks functional RB and the AR to study the functional relationships between these two regulators. Although previous experiments have established DU145-derived cell lines with constitutive re-expression of RB (suggesting that RB did not block cell cycle progression in DU145 cells; Ref. 4), we still used inducible expression to avoid possible selection for cells that contain mutations that abolish the negative effects of RB during the generation of derivative cell lines. We transfected DU145 cells with pUHD-172neo to establish clonal lines that could express a test gene under the control of the tet-responsive promoter in the vector pUHD10–3 in response to Dox. Five such clones (DU-rtTA) were found to have this ability (data not shown).

DU145 cells are microsatellite mutator phenotype positive (16). Defects in DNA mismatch repair in microsatellite mutator phenotype positive cells render them prone to replication errors. The human Bax gene contains a sequence of eight consecutive deoxyguanosines (the G-8 track) near its NH_2 terminus, which is prone to mutations caused by DNA slippage during DNA replication. It was reported that the G-8 track of the Bax gene is homozygously mutated to G-9, and consequently the Bax protein is not expressed in DU145 cells (16). In theory, the instability at the 8-G track should generate both wild type-to-mutant and mutant-to-wild type changes if no selective pressure is present to select against Bax-expressing cells. We determined Bax expression in the five DU-rtTA clones. As shown in Fig. 1*A*, two of the five clones have restored Bax expression. We did not detect any difference in the proliferation rates among all these clones compared with the parental cells (data not shown). We picked clone 1 (the

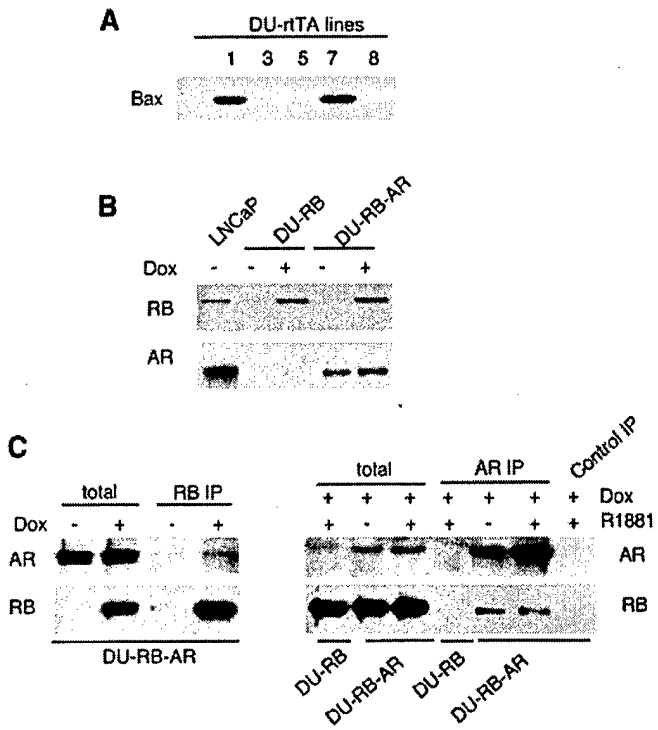

Fig. 1. Restoration of Bax, retinoblastoma (RB), and androgen receptor (AR) expression in DU145 prostate cancer cells and physical interaction between RB and the AR. *A*, total cell extracts from parental DU145 and five DU-rtTA clonal lines were Western blotted with an anti-Bax antibody. *B*, equal amounts of total cell extracts from LNCaP, DU-RB, and DU-RB-AR cell lines were Western blotted with antibodies against RB and the AR. RB expression was induced by the addition of doxorubicin (Dox) in the culture media for 16 h. *C*, total extracts of DU-RB and DU-RB-AR cells, uninduced or induced by Dox as indicated, were either analyzed directly by Western blotting (the "total" lanes) or immunoprecipitated first with RB or AR antibodies [the "RB IP" and "AR IP" lanes]. In the *left panel*, the cells were incubated in media containing fetal bovine serum, whereas in the *right panel*, cells were incubated in media containing charcoal dextran-treated serum (CDT) with or without the added synthetic androgen R1881 at 1 nM for 48 h. *IP*, immunoprecipitation.

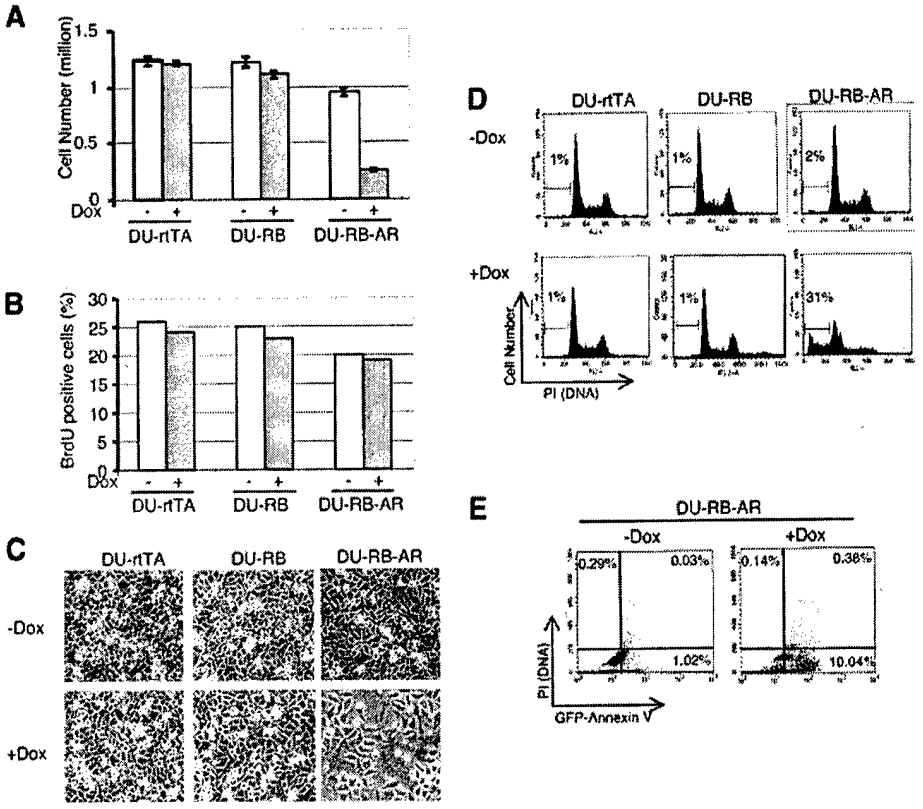

Fig. 2. Apoptosis caused by re-expression of retinoblastoma (RB) and the androgen receptor (AR) in DU145 cells. DU-rtTA, DU-RB, and DU-RB-AR cells were treated with doxorubicin (Dox), or untreated in parallel, as indicated, for 24 h. Total numbers of attached cells were counted (*A*) after the plates were photographed under a phase-contrast microscope (*C*). Cells were pulse-labeled with BrdUrd for 4 h, fixed, stained with anti-BrdUrd, and the number of BrdUrd-positive cells determined (*B*). Total cells (including the floating cells) were fixed in ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry (*D*). Percentages of cells in the sub-G_1 region were indicated. Total cells were stained with green fluorescent protein-Annexin V and PI without ethanol fixation and analyzed by flow cytometry (*E*). Cells in the *bottom right window* were defined as apoptotic because they were Annexin V-stained positive but PI-stained negative (PI did not leak through the cell membrane of live cells). Cells in the *top left window* were defined as death through necrosis. Cells appeared in the *top right window* in the late stages of apoptosis because of secondary cell membrane damage.

DU-rtTA cells) to establish subsequent cell lines and continued to monitor Bax expression. Bax expression was retained in all of the subsequent clones in this study (data not shown).

We then transfected the DU-rtTA cells with pUHD10–3-RB (13) to establish clones that could be induced to express RB. A total of four independent cell lines were established and were found to exhibit similar properties. Data from a representative one, DU-RB (Fig. 1*B*), were chosen for presentation in this report. From the DU-RB cells, we further derived lines that contained constitutive expression of the AR by stably transfecting DU-RB cells with pcDNA3-ARzeo to create a pair of DU145 cells (DU-RB-AR cells; Fig. 1*B*) that only re-express the AR (DU-RB-AR cells without induction of RB) and that re-express both the AR and RB (DU-RB-AR cells with induction of RB). Again, one representative clone of three was presented in this report. When equal amounts of total proteins were compared, the AR and Dox-induced RB levels in DU-RB-AR cells were about 3-fold lower and 2-fold higher, respectively, than those in the LNCaP cells, which express functional RB and the AR and are androgen responsive and androgen dependent for proliferation (Fig. 1*B*).

Previous studies have demonstrated that RB and the AR could physically interact *in vitro* with purified RB and the AR proteins, and *in vivo* in mammalian two-hybrid assays (11, 12). RB interacted with the NH_2-terminal part of the AR, and this interaction was independent on the AR ligand. With our inducible cell lines, we could now demonstrate *in vivo* interaction of RB and the AR through coimmunoprecipitation (Fig. 1*C*). This interaction was also independent on the AR ligand. These results suggest a direct relationship between RB and the AR in DU145 cells.

Combined Re-expression of RB and the AR Induces Apoptosis. Inducible re-expression of RB (DU-RB cells with Dox) did not result in inhibition of cell proliferation compared with uninduced cells in parallel, as measured by cell number determination, BrdUrd labeling, and cell morphology (Fig. 2, *A–C*). DU145 cells with constitutive re-expression of the AR (DU-RB-AR cells without induction of RB) showed a slight reduction in proliferation activity compared with the parental cells as measured by cell number determination and BrdUrd labeling (Fig. 2, *A* and *B*). We further determined whether re-expression of the AR, in the absence of RB induction, rendered DU145 cells androgen responsive by comparing their proliferation after 2 days of culture in media containing charcoal dextran-treated FBS (CDT media) and CDT media containing 1 nM DHT. BrdUrd labeling at the end of the 2 day period showed same labeling indices for media containing FBS, CDT, or CDT + DHT (data not shown). These results demonstrate that re-expression of RB or the AR alone did not have significant effects on DU145 cell proliferation in the conditions used.

We then studied the effects of re-expressing RB together with the AR by inducing DU-RB-AR cells in FBS media. Twenty-four h after RB induction, we observed a significant reduction in the numbers of attached cells compared with uninduced cultures in parallel (Fig. 2, *A* and *C*). This reduction in cell numbers was apparently not caused by a block to enter S phase as measured by BrdUrd incorporation (Fig. 2*B*) but by cell death through apoptosis. As shown in Fig. 2*D*, RB induction in DU-RB-AR cells resulted in the appearance of sub-G_1 cell fractions in flow cytometry analysis and the externalization of phosphatidylserine on the plasma membrane as measured by Annexin V staining (Fig. 2*E*). Clearly, the combined re-expression of RB and the AR created an apoptotic activity, which was not present when RB or the AR was re-expressed alone.

RB + AR Affects Mitochondria Integrity to Induce Apoptosis. Apoptoic mechanisms can be generally divided into mitochondria-dependent and -independent groups. To determine the involvement of mitochondria in RB + AR-induced apoptosis, we measured the mitochondria membrane integrity by $DiOC_6$ staining. $DiOC_6$ is selectively retained in the mitochondria when the mitochondria membrane potential is intact. Fig. 3*A* shows that re-expression of RB led to the loss of mitochondria retention of $DiOC_6$. The extent of this loss was

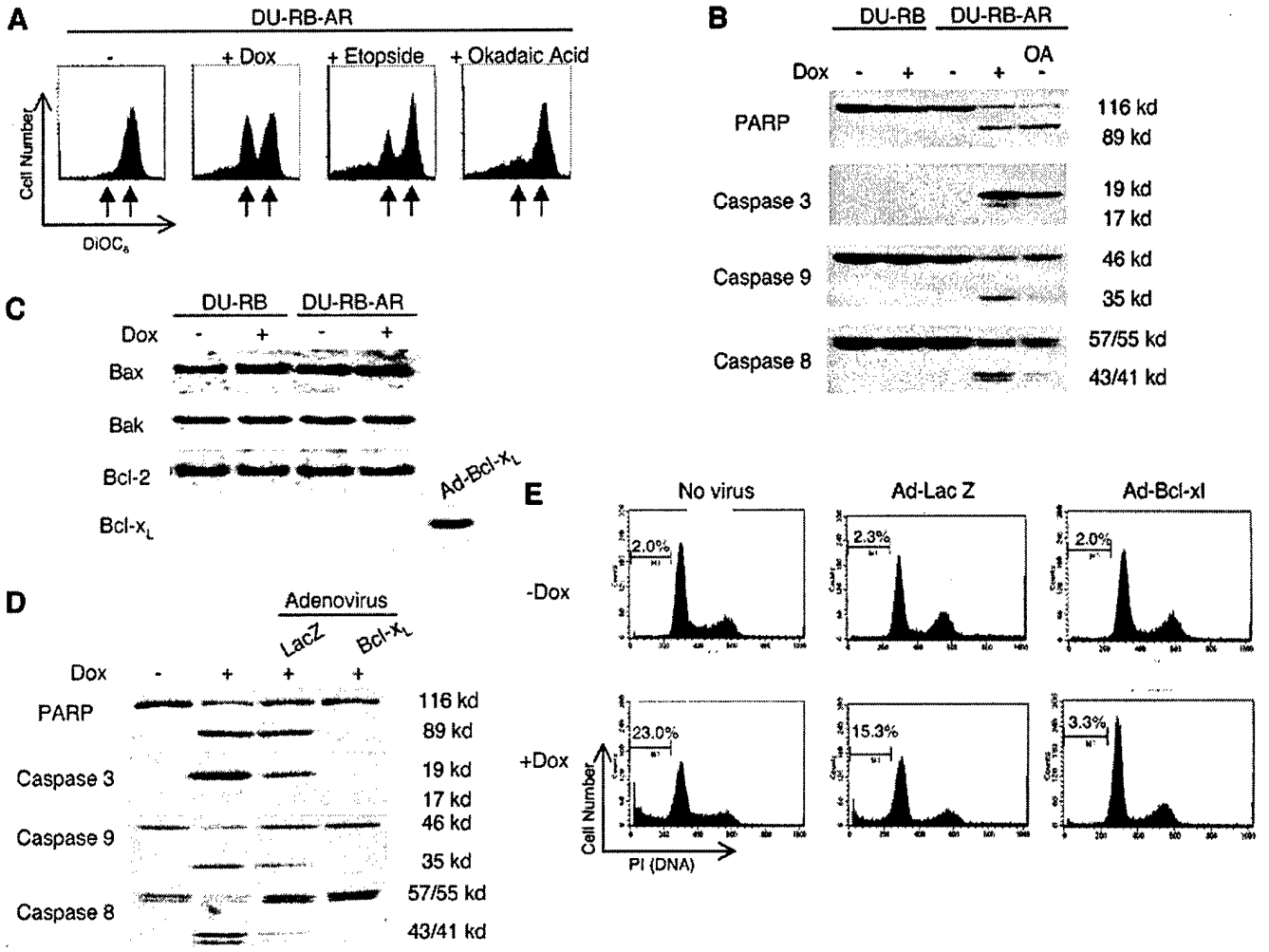

Fig. 3. Involvement of the mitochondria-initiated caspase activation pathway in retinoblastoma (RB) + androgen receptor (AR)-induced apoptosis. *A*, DU-RB-AR cells were induced with doxorubicin (Dox), or treated with the indicated apoptosis-inducing chemicals without Dox, for 24 h. Total cells were then incubated with $DiOC_6$ for 30 min and analyzed by flow cytometry. Cells in the lower-brightness peak contained damaged mitochondria. *B*, DU-RB and DU-RB-AR cells were treated with Dox, or okadaic acid in the absence of Dox, as indicated, for 24 h. Cell extracts were made from total cells and analyzed by Western blotting with the indicated antibodies. Molecular weights of full-length and cleaved caspases are marked. *C*, DU-RB and DU-RB-AR cells were induced with Dox as indicated, and protein levels of Bcl-2, Bax, and Bcl-x_L in total extracts were determined with Western blotting with the respective antibodies. Expression of Bcl-x_L after infection of DU-RB-AR cells with a Bcl-x_L-expressing adenovirus was also shown. *D*, DU-RB-AR cells were first infected with adenoviruses expressing either LacZ or Bcl-x_L for 24 h and then induced with Dox for another 24 h. Western blotting was performed as in *B*. *E*, DU-RB-AR cells were treated as in *D*, harvested, and analyzed for sub-G_1 fraction as in Fig. 2*D*. *PARP*, poly(ADP-ribose) polymerase; *OA*, okadaic acid.

greater than those induced by chemicals that are widely used to induce mitochondria-mediated cell death such as etopside and okadaic acid. As shown later in Fig. 6*B*, induction of RB in DU-RB cells with Dox did not cause mitochondria damage.

We next determined the activation status of several key caspases by their cleavage and the cleavage of the caspase substrate poly(ADP-ribose) polymerase. As shown in Fig. 3*B*, RB induction caused significant cleavage of poly(ADP-ribose) polymerase, caspase 3, 9, and 8. The extent of these effects was similar to those induced by okadaic acid. The activation of caspases 9 and 3 are consistent with the well-established mitochondria-initiated caspase activation pathway (mitochondria damage → cytochrome C release → caspase 9 cleavage → caspase 3 cleavage). The cleavage of caspase 8 could be mediated by other activated caspases (including caspases 9 and 3). Alternatively, caspase 8 cleavage could be caused by the activation of the death receptor pathway or other mechanisms upstream of mitochondria. The Bcl-2 family members Bcl-2, Bcl-x_L, Bax, and Bak are the major regulators of mitochondria integrity (17). Bcl-2 and Bcl-x_L protect mitochondria membrane integrity whereas Bax and Bak induce mitochondria membrane depolarization. Bcl-2, Bax, and Bak protein levels did not change detectably after RB induction in both DU-RB and DU-RB-AR cells; but Bcl-x_L proteins were not detectable (Fig. 3*C*). We therefore ectopically expressed Bcl-x_L through an adenovirus vector in DU-RB-AR cells to determine the role of mitochondria damage in the cleavage of caspase 8 and apoptosis after RB induction. As shown in Fig. 3*D*, adenovirus-mediated expression of Bcl-x_L completely blocked cleavage of caspase 8 as well as caspases 9 and 3. Bcl-x_L also completely prevented apoptosis (Fig. 3*E*). In the same experiments, the control LacZ adenovirus only slightly reduced caspase activation and sub-G_1 fraction. We conclude from these results that RB + AR caused mitochondria damage to induce apoptosis.

RB + AR-Induced Apoptosis Requires Functions of the Pro-apoptosis Proteins Bax and Bak but Is Independent on p53. Well-established mediators of mitochondria damage include the pro-apoptosis members of the Bcl-2 family Bax and Bak. These two proteins can self-oligomerize in mitochondria outer membrane to permeabilize it (18). In the mouse, knockout of both Bax and Bak

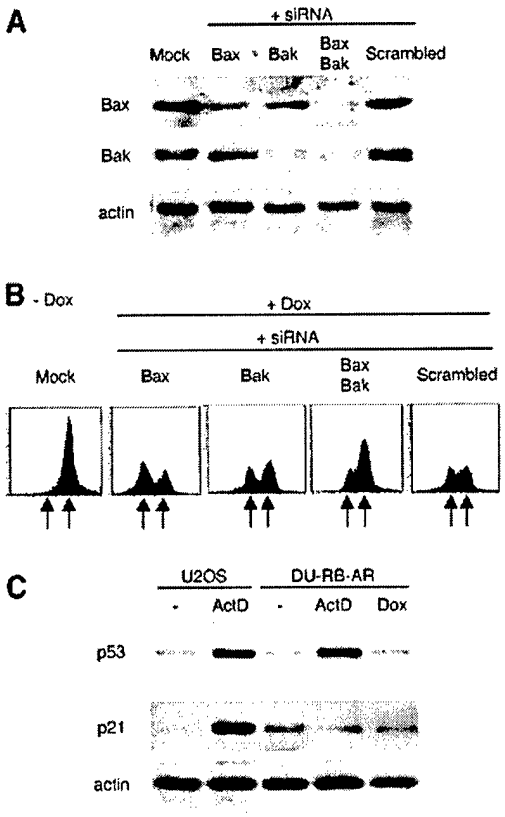

Fig. 4. Roles of the proapoptotic proteins Bax and Bak, and p53 in retinoblastoma (RB) + androgen receptor (AR)-induced apoptosis. *A*, DU-RB-AR cells were transfected with the indicated siRNA, induced with doxorubicin (Dox), and then analyzed by Western blotting to determine the protein levels of Bax, Bak, and actin. In *B*, DU-RB-AR cells treated in parallel were analyzed by $DiOC_6$ labeling as in Fig. 3*A*. *C*, U2OS and DU-RB-AR cells were treated with actinomycin D (20 nM) or Dox as indicated for 24 h. Total cell extracts were then analyzed by Western blotting to determine the levels of p53, p21, and actin. *siRNA*, small interfering RNA

conferred resistance to normal apoptosis in animal development and in experimental apoptosis induced by many apoptosis-inducing agents, whereas knockout of Bax or Bak alone did not (19, 20). In human colon cancer cells, knockout of Bax alone resulted in complete resistance to apoptosis induced by non-steroidal anti-inflammatory drugs but no change in 5-fluorouracil and ceramide-induced apoptosis (21). Lack of Bax expression was also shown to favor clonal selection in tumor growth when engrafted in nude mice (22). The status and role of Bak were not addressed in these studies. The parental DU145 cells do not express Bax because of a frame-shift mutation, which was corrected during the establishment of our inducible cell lines (Fig. 1*A*).

We used siRNA-mediated knockdown to determine the roles of Bax and Bak in RB + AR-induced apoptosis, as shown in Fig. 4, *A* and *B*. Knockdown of Bax or Bak (by >75%) did not affect the ability of RB + AR to induce apoptosis, suggesting that RB + AR-induced apoptosis is not dependent on Bax or Bak alone. When both Bax and Bak were subjected to knockdown, RB + AR-induced apoptosis was almost completely prevented (Fig. 4*B*). Thus, the apoptosis pathway activated by RB + AR involves Bax and Bak functions, which is consistent with our finding that ectopic expression of Bcl-x_L was able to prevent RB + AR-induced apoptosis (Fig. 3) because Bcl-x_L antagonizes the activity of Bax and Bak.

We next determined whether RB + AR-induced apoptosis was dependent on p53. p53 of DU145 cells contains two point mutations Pro274-to-Leu and Val223-to-Phe (23). To determine the functional status of this mutant p53, we treated DU-RB-AR cells with actinomycin D. Cells containing functional p53 (U2OS) were studied in parallel. As shown in Fig. 4*C*, actinomycin D treatment induced p53 protein levels in both DU-RB-AR and U2OS cells, indicating that the signaling pathway to p53 and the ability of the mutant p53 in DU-RB-AR cells to stabilize are intact. However, the mutant p53 in DU-RB-AR cells was completely defective in its ability to transactivate the well-established p53 target gene p21Cip1 in response to actinomycin D treatment (Fig. 4*C*). These results suggest that RB + AR is able to induce apoptosis in the absence of a functional p53.

Biochemical Effects of RB and the AR on Each Other. RB and the AR both have well established biochemical activities in regulation of gene expression. We investigated how RB and the AR affected the activities of each other in the prostate cancer DU145 cells. The best established function of RB is the repression of E2F. We determined the effects of RB induction on an E2F reporter in the absence (DU-RB cells) and presence (DU-RB-AR cells) of the AR; in the latter case, we also determined the effects of the AR ligand DHT. As shown in Fig. 5*A*, induction of RB repressed the E2F reporter activity about 30% in DU-RB cells and about 50% in DU-RB-AR cells. The repression was not influenced by the presence or absence of the AR ligand DHT in DU-RB-AR cells in CDT media. When RB was expressed from transient transfection, which produced higher levels of RB proteins, repression of the E2F reporter was to a greater extent at about 60% in DU-RB cells and 50–60% in DU-RB-AR cells in the presence or absence of DHT. When an unphosphorylable RB was expressed by transient transfection, it led to further greater repression of E2F in

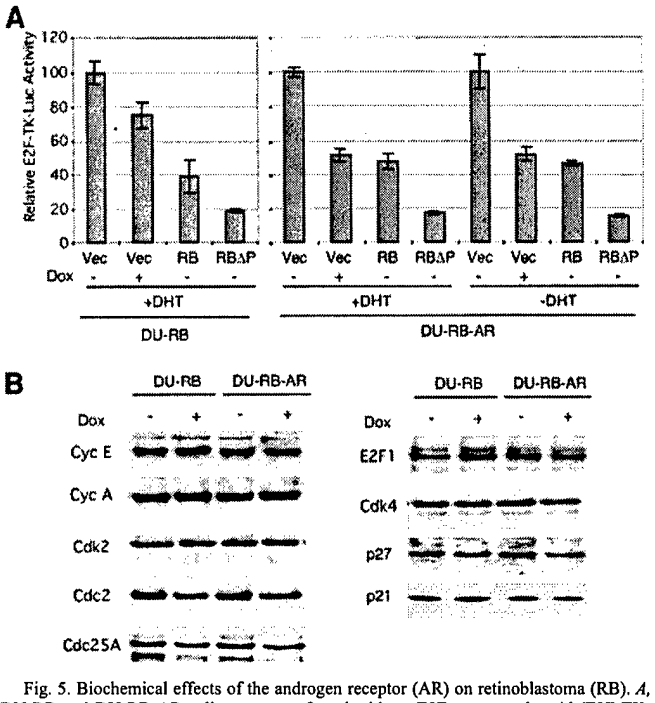

Fig. 5. Biochemical effects of the androgen receptor (AR) on retinoblastoma (RB). *A*, DU-RB and DU-RB-AR cells were transfected with an E2F-reporter plasmid (E2F-TK-Luc) together with the indicated expression vectors. RBΔP is an unphosphorylable RB with phosphorylation sites mutated. After transfection, the cells were incubated in charcoal dextran-treated serum media with dihydrotestosterone (DHT; 1 nM) or vehicle (ethanol), and with or without doxorubicin (Dox) as indicated. Luciferase activity was measured and presented as relative units. Triplicate samples were analyzed for every data point and SDs displayed as *error bars*. The experiments were repeated three times with similar results. *B*, total cell extracts of DU-RB and DU-RB-AR cells, with or without induction for 18 h, were analyzed with Western blotting with the indicated antibodies. *Vec*, empty vector; *Cyc*, cyclin; *Cdc2*, cyclin-dependent kinase 1; *Cdk*, cyclin-dependent kinase.

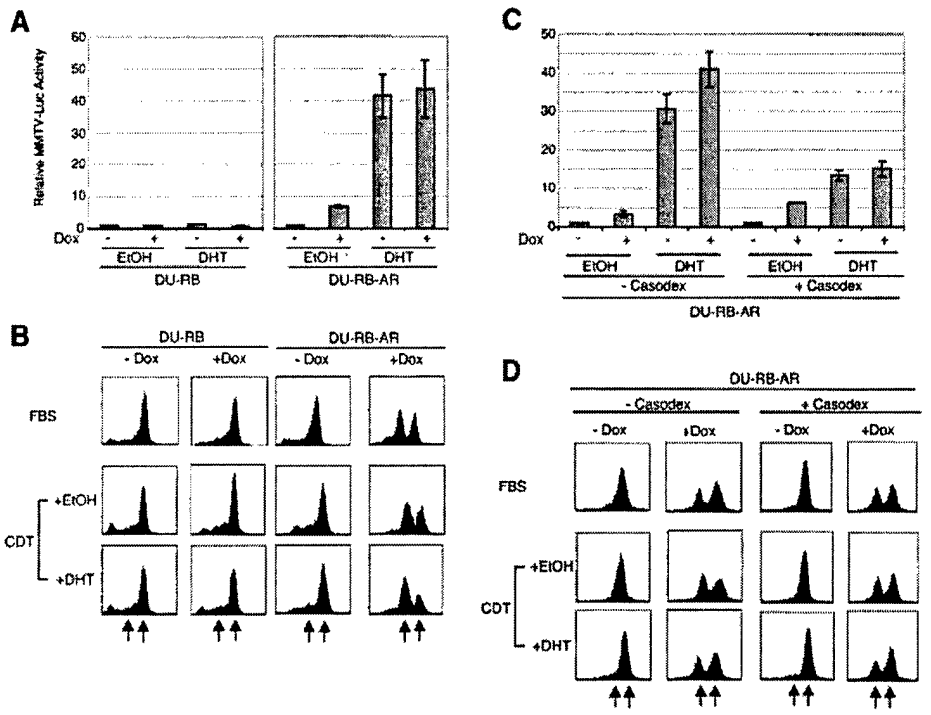

Fig. 6. Biochemical effects of retinoblastoma (RB) on the androgen receptor (AR). *A*, DU-RB and DU-RB-AR cells were transfected with an AR reporter plasmid [mouse mammary tumor virus (MMTV)-AR] and were incubated in charcoal dextran-treated serum (CDT) media containing dihydrotestosterone (DHT; 1 nM) or vehicle (ethanol) and with or without doxorubicin (Dox). Luciferase activity was measured and presented as relative units. Triplicate samples were analyzed for every data point and SDs displayed as *error bars*. The experiments were repeated three times with similar results. *B*, DU-RB and DU-RB-AR cells were incubated in different media as indicated and induced with Dox for 24 h. Cells were then stained with $DiOC_6$ and analyzed as in Fig. 3*A*. Experiments in *C* and *D* were performed in identical ways as in *A* and *B*, respectively, but including a parallel group with the treatment of Casodex (20 μM) as indicated. *EtOH*, ethanol; *FBS*, fetal bovine serum.

both cell lines. This pattern of E2F repression by wild type and unphosphorylable RB in DU145-derived cell lines is similar to what has been observed after ectopic expression of RB in many other cell lines that are not sensitive to the G_1-S arresting functions of RB.

We also determined the effects of RB-mediated E2F repression on cellular genes. As shown in Fig. 5*B*, proteins levels of several well-established RB-E2F repression target genes such as cyclin E, cyclin A, Cdk2, Cdc2, Cdc25A, and E2F1 itself were not detectably affected by the induction of RB in the absence or presence of the AR. Protein levels of Cdk4 and the cyclin-dependent kinase inhibitors p27 and p21 were also not affected by RB and the AR in these cells. The failure of induced RB to repress cellular E2F target genes could be caused by a number of factors (including deficiencies in histone deacetylases and/or chromatin remodeling proteins), and could be the reason for the inability of RB to cause G_1-S block in these cells [(4) and Fig. 2*B*]. Nonetheless, data in Fig. 5 suggest that coexpression of the AR did not affect RB's regulation of E2F in DU145 cells.

We measured the AR transactivation activity with the natural AR-responsive mouse mammary tumor virus-Luc reporter. As shown in Fig. 6*A*, the AR in DU-RB-AR cells responded to DHT to similar extents as previous reports with transiently transfected AR in DU145 cells and in our DU-RB cell line (data not shown). Importantly, coexpression of RB in CDT media without added DHT significantly stimulated the transactivation activity of AR in the same assay. This stimulation of mouse mammary tumor virus-Luc was dependent on the AR because RB induction in DU-RB cells did not result in this stimulation. Dox also had no stimulatory effect on AR transactivation in the absence of RB (data not shown). Casodex, an androgen antagonist, inhibited DHT-induced AR transactivation activity by about 50%, which is consistent with reports in the literature (24, 25) but did not reduce RB-induced AR transactivation activity in CDT media without added DHT (Fig. 6*C*), providing further support that RB can induce AR transactivation activity in the absence of androgen. When RB was induced in the presence of DHT, it did not affect DHT-stimulated AR transactivation activity.

The above results from inducible cell lines differed from pervious results obtained with transient transfection of the AR and RB, in which RB only stimulated AR transactivation activity in the presence of DHT (11, 12). Indeed, when we used transient transfection to express the AR and RB, we also found that RB only stimulated the AR

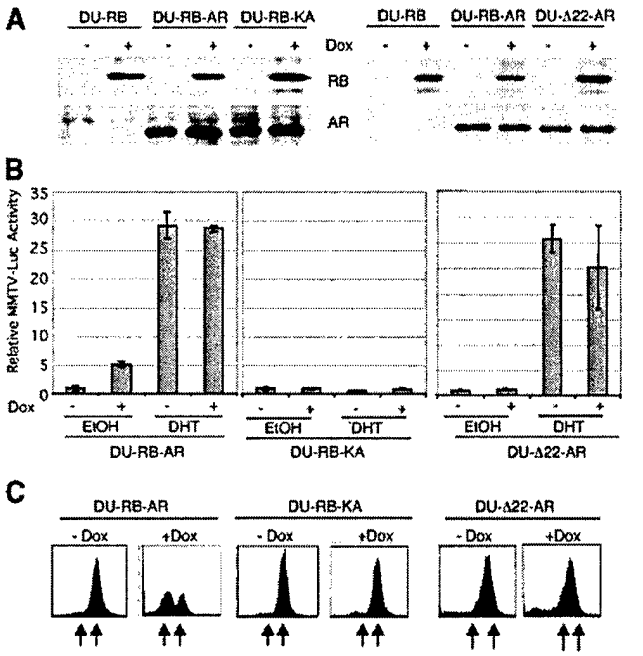

Fig. 7. Requirement for the transactivation activity of the androgen receptor (AR) and the pocket functions of retinoblastoma (RB) in RB + AR-induced apoptosis. *A*, Western blotting of total cell extracts of DU-RB, DU-RB-AR, DU-RB-KA (expressing AR-K630A mutant), and DU-Δ22-AR (expressing RBΔ22 mutant) cells with anti-RB and anti-AR antibodies as in Fig. 1*B*. *B*, DU-RB-AR, DU-RB-KA, and DU-Δ22-AR cells were transfected with the AR reporter plasmid mouse mammary tumor virus (MMTV)-AR and Luciferase activity analyzed as in Fig. 6*A*. *C*, DU-RB-AR, DU-RB-KA, and DU-Δ22-AR cells cultured in fetal bovine serum media were induced with doxorubicin (Dox) for 24 h and analyzed with $DiOC_6$ staining as in Fig. 3*A*. *EtOH*, ethanol.

in the presence of DHT. This difference between results from stable inducible cell lines and transient transfection is most likely caused by the different expression levels in these different experimental systems.

To determine whether the stimulation of AR transactivation activity by RB in the absence of added DHT was functionally relevant to the RB + AR-induced apoptosis, we measured mitochondria integrity after induction of RB in this condition. Fig. 6*B* shows that RB induction in CDT media caused apoptosis to the same extent as in the FBS media. Addition of DHT only slightly increased apoptosis further. Fig. 6*D* shows that addition of Casodex did not reduce RB + AR-induced mitochondria damage. Therefore, the stimulation of AR transactivation activity by RB in CDT media was sufficient for the induction of apoptosis. It is important to note here that DHT induced greater AR transactivation activity than RB but did not induce apoptosis in the absence of re-expressed RB.

AR Transactivation Activity and RB Pocket Functions Are Required for RB to Induce Apoptosis. We used a genetic approach to determine whether the activation of the transactivation activity of the AR was required for RB to induce apoptosis. It was recently demonstrated that a K630-to-A mutation at the acetylation consensus site abrogated the transactivation activity of the AR (26). We transfected the same DU-RB cell line with a pCDNA3-AR-KAzeo construct and selected with zeocin for clones with AR-KA expression, as we did for the DU-RB-AR cell line. A representative cell line (called DU-RB-KA for brevity) was shown in Fig. 7*A*. Expression levels of the AR-KA protein in this cell line were the same as the levels of the wild-type AR expressed in the DU-RB-AR cell line, so were the levels of RB expression induced by Dox. As expected, the AR-KA in this cell line was unable to transactivate the mouse mammary tumor virus-Luc reporter in response to DHT (Fig. 7*B*). It also failed to be stimulated by RB induction. Induction of RB in this cell line did not induce mitochondria damage (Fig. 7*C*) and DNA fragmentation (data not shown). We conclude that the transactivation activity of the AR is required for RB to induce apoptosis.

Most of the known activities of the RB protein are mediated through its pocket domain, and most of the naturally occurring mutations of RB map inside the pocket domain and disrupt its function. We established parallel cell lines expressing RBΔ22, the pocket functions of which are abolished by deletion of exon 22, to determine the relationship of RB + AR-induced apoptosis with the pocket domain. In this cell line (DU-Δ22-AR), protein levels of RBΔ22 after induction and the AR were similar to those in the DU-RB-AR cell line (Fig. 7*A*). As shown in Fig. 7*B*, induction of RBΔ22 did not activate AR transactivation activity and (Fig. 7*C*) did not induce apoptosis, demonstrating that the RB + AR-induced apoptosis is dependent on the pocket functions of RB. This result is consistent with previous findings that binding of viral onco-proteins (E1a, Tag, and E7) to the pocket domain blocked RB-AR interaction (12). This result suggests that the ability of RB to activate the AR to induce apoptosis may be involved in the roles of RB as a tumor suppressor in prostate cancer cells.

DISCUSSION

Although counter intuitive to the role of RB as a tumor suppressor, RB has been believed to have an antiapoptotic activity in a number of tissues based on phenotypes of the RB knockout mice (27–29). Importantly however, recent studies with more advanced mouse genetic techniques have demonstrated that the apoptosis observed in RB knockout mouse embryos could largely be attributed to non-cell-autonomous effects of inactivating RB in the whole animal. Perhaps most striking is the demonstration that most of the defects observed previously in RB knockout embryos could be prevented by supplying the RB knockout embryo with normal placenta (30). It was shown that RB inactivation led to increased proliferation, not apoptosis, of trophoblasts resulting in defective nutrient transport from the mother to the embryo. These recent findings clearly indicate the need to re-evaluate previous interpretations of the apoptosis phenotypes in various tissues of RB knockout embryos.

In this study we identified an apoptotic activity of RB by re-expressing RB together with the AR in a prostate cancer cell model that is deficient for both RB and the AR. This functional identification significantly improves our knowledge of RB in prostate cells obtained from a large amount of previous studies of this subject.

Roles of RB and the AR in Prostate Cell Apoptosis *in Vivo*. A well-known physiological apoptotic response in prostate epithelial cells takes place after castration (31), indicating that androgen-AR signaling is antiapoptotic whereas the non- or low-androgen-stimulated AR may be apoptotic. It was shown that immediately before the onset of apoptosis, RB expression increased significantly in prostate epithelial cells, implicating a proapoptosis role of RB in prostate epithelial cells (32). Reconstitution of prostate tissue with RB knockout prostate epithelial cells in an otherwise RB wild-type host revealed that RB inactivation led to hyperplasia of prostate epithelium, not apoptosis (33). When these animals were subjected to pharmacological doses of androgen and estrogen, RB-deficient prostate tissues are more prone to oncogenic transformation without increases in apoptosis, providing genetic evidence for an antiproliferative, but not antiapoptotic, role of RB in prostate epithelial cells (33). On the other hand, transgenic expression of the AR in prostate epithelial cells through a probasin promoter in an otherwise wild-type mouse led to both proliferation and apoptosis resulting in no net hyperplasia, demonstrating that the AR signaling can promote both proliferation and apoptosis (34). Precancerous nodules were observed only in focal regions indicating that additional mutations, most likely those that abolish apoptosis, are necessary for oncogenic transformation. RB mutation could be one such additional mutation, which would be consistent with the presence of RB mutations in a significant portion of prostate cancers in humans. Our discovery that the combined action of RB and the AR can lead to apoptosis may provide a mechanistic explanation for these *in vivo* observations and a cell model to study the apoptotic roles of RB and the AR in prostate cells.

Roles of RB and the AR in Prostate Cell Apoptosis *in Vitro*. The roles of RB and the AR in apoptosis have been separately studied in cultured prostate cancer cells. The AR has been shown to play a proapoptosis role in prostate cancer cells in a number of scenarios. When re-expressed in the prostate cancer cell line PC-3 (which contains RB), androgen could induce apoptosis (35). Brca1 could further stimulate the androgen-activated AR transactivation activity to enhance apoptosis in these cells (36). The AR has also been shown to play an apoptotic role in prostate cancer cells in the absence of coexpressed RB. Overexpression of activated MEKK1 (mitogen-activated protein kinase kinase kinase 1) required the AR to cause apoptosis in DU145 cells (25). Like Brca1, MEKK1 stimulated AR transactivation activity and induced apoptosis in an androgen-dependent manner. These studies have led to the current belief that "super-activation" of the AR can cause apoptosis. A unique feature of RB + AR-induced apoptosis demonstrated in our study is that RB could activate AR transactivation activity and induce apoptosis in media containing charcoal dextran-treated (CDT) serum without added androgen. More importantly, we showed that androgen-stimulated AR transactivation activity to a greater degree than RB but did not induce apoptosis in the absence of coexpressed RB. Thus, it is unlikely that RB-induced apoptosis by super-activating AR transactivation activity. This characteristic of RB + AR-induced apoptosis is more consistent with the currently known *in vivo* prostate cell apo-

ptosis as discussed above. Pharmacological doses of androgen caused hyperplasia, but not apoptosis, in the reconstituted RB−/− prostate tissues (33); prostate cell apoptosis occurred without the need for high dose of androgen in probasin-AR transgenic mice (34), and prostate epithelial cells underwent apoptosis after castration (androgen withdrawal; Ref. 31).

Overexpression of RB in cultured cells generally leads to inhibition of cell cycle progression but caused apoptosis in prostate cancer LNCaP cells (32), which contain RB and the AR. It was also proposed that RB might be required for apoptosis of prostate cancer cells induced by cell detachment and protein kinase C activation because these treatments led to caspase activation and apoptosis in LNCaP cells but not in DU145 cells (37). Interestingly however, it is known that fibroblasts respond to these same treatments with G_1 cell cycle arrest, not apoptosis. The reason for these differences has not been understood. Based on our finding, we suggest that the presence of the AR signaling pathway in prostate cells could be responsible for these differences.

In another study, it was shown that constitutive re-expression of RB alone in DU145 cells sensitized these cells to killing by γ-irradiation (38, 39). Interestingly, this cell killing in the absence of the coexpressed AR did not involve caspase activation. Rather, a serine protease was involved. We have demonstrated that RB + AR-induced apoptosis in DU145 cells is through mitochondria-initiated caspase pathway, a cell death pathway activated in most, if not all, apoptotic responses to many signals including DNA damage, oncogenic stimuli, and disruptions to metabolism and intracellular trafficking. Our findings that RB stimulated AR transactivation and apoptosis in the absence of added androgen; androgen stimulated AR transactivation activity to a greater extent but did not induce apoptosis in the absence of RB; and RB did not induce apoptosis when co-re-expressed with a transactivation-defective AR mutant suggest that RB may stimulate the AR to activate expression of a set of genes to induce apoptosis, and this set of genes are different from those activated by the AR and androgen. Currently, almost all studies of cellular AR target genes have been done with the prostate cancer cell line LNCaP (40, 41), because this cell line shows androgen responsiveness for proliferation and expression of the well-established AR target gene prostate-specific antigen (42). The kinase inhibitor p21Cip1 has been recently demonstrated as a direct AR target gene in LNCaP cells (43), but conflicting results were obtained in another study with the same LNCaP cells (44). It is clear that many key AR target genes remain to be identified. A major difficulty in identifying AR target genes is that although ectopically expressed AR is functional as measured by Androgen receptor Response Element (ARE)-containing promoter reporter plasmids, it generally does not lead to androgen responsiveness of the currently known cellular AR target genes. This is indeed the case with AR re-expression in DU145 cells. The AR target gene prostate-specific antigen responded robustly to androgen in LNCaP cells but was not detectably stimulated by androgen in the DU-RB-AR cells under the same conditions (our unpublished results). This phenomenon indicates that a lot remains to be learned about the cofactors that are involved in AR-mediated transcription regulation of cellular genes. RB may serve as one cofactor to stimulate AR transactivation activity for a specific set of genes that regulate the mitochondria-mediated apoptotic pathway.

Underlying Mechanisms for the Opposing Effects of the AR in Apoptosis. Increased apoptosis of prostate epithelial cells after castration perhaps represents the most physiologically relevant evidence that the AR can play opposing roles in the regulation of prostate cell survival, because this phenomenon suggests that androgen-AR signaling is antiapoptotic whereas the non- or low-androgen-stimulated AR is apoptotic. How, mechanistically, could the same AR play opposing roles in prostate cell survival? Re-expression of the tumor suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10) in LNCaP cells causes apoptosis, which is countered by androgen-AR signaling (45). In this system, it was demonstrated that the androgen-activated AR represses the forkhead transcription factor FKHR (the target genes of which include proapoptotic proteins such as the Fas ligand and Bim) to inhibit apoptosis. Castration also induces increased apoptosis in vertebral osteoblasts (46). With the osteoblasts (and extended to mouse embryo fibroblasts and human HeLa cells), Kousteni *et al.* (46) reported that both androgen and estrogen receptors have sex-nonspecific ligand-dependent antiapoptotic activity, which depends on a cytoplasmic function of the receptors to stimulate Src/Shc/Erk signaling. Based on these studies, it appears that the antiapoptotic activity of androgen-AR signaling may not depend on the transactivation activity of the AR. In this study, we showed that the RB + AR-induced apoptosis is dependent on the transactivation activity of the AR (as measured on a adenine and uridine-rich element promoter reporter). These findings together suggest that the opposing effects of the AR in prostate cell survival, at least in the experimental scenarios used, are based on distinct molecular activities of the AR.

ACKNOWLEDGMENTS

We thank Drs. Mark Czaja, Hermann Bujard, J. Wade Harper, Kristian Helin, Rick Kitsis, Jeffery Molkentin, and Richard Pestell for reagents and advice.

REFERENCES

1. Classon, M., and Harlow, E. The retinoblastoma tumour suppressor in development and cancer. Nat. Rev. Cancer, *2:* 910–917, 2002.
2. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.
3. Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57–70, 2000.
4. Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P., and Lee, W-H. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science (Wash. DC), *247:* 712–715, 1990.
5. Phillips, S. M., Barton, C. M., Lee, S. J., Morton, D. G., Wallace, D. M., Lemoine, N. R., and Neoptolemos, J. P. Loss of the retinoblastoma susceptibility gene (RB1) is a frequent and early event in prostatic tumorigenesis. Br. J. Cancer, *70:* 1252–1257, 1994.
6. Kubota, Y., Fujinami, K., Uemura, H., Dobashi, Y., Miyamoto, H., Iwasaki, Y., Kitamura, H., and Shuin, T. Retinoblastoma gene mutations in primary human prostate cancer. Prostate, *27:* 314–320, 1995.
7. Brooks, J. D., Bova, G. S., and Isaacs, W. B. Allelic loss of the retinoblastoma gene in primary human prostatic adenocarcinomas. Prostate, *26:* 35–39, 1995.
8. Tilley, W. D., Buchanan, G., Hickey, T. E., and Bentel, J. M. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin. Cancer Res., *2:* 277–285, 1996.
9. Koivisto, P., Kolmer, M., Visakorpi, T., and Kallioniemi, O. P. Androgen receptor gene and hormonal therapy failure of prostate cancer. Am. J. Pathol., *152:* 1–9, 1998.
10. Culig, Z., Hobisch, A., Hittmair, A., Peterziel, H., Cato, A. C., Bartsch, G., and Klocker, H. Expression, structure, and function of androgen receptor in advanced prostatic carcinoma. Prostate, *35:* 63–70, 1998.
11. Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, C., Su, C., and Chang, C. Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. Biochem. Biophys. Res. Commun., *248:* 361–367, 1998.
12. Lu, J., and Danielsen, M. Differential regulation of androgen and glucocorticoid receptors by retinoblastoma protein. J. Biol. Chem., *273:* 31528–31533, 1998.
13. Jiang, H., Karnezis, A. N., Tao, M., Guida, P., and Zhu, L. pRB and p107 have distinct effects when expressed in pRB-deficient tumor cells at physiologically relevant levels. Oncogene, *19:* 3878–3887, 2000.
14. Jiang, H., Chou, H. S., and Zhu, L. Requirement of cyclin E-Cdk2 inhibition in $p16^{INK4a}$-mediated growth suppression. Mol. Cell. Biol., *18:* 5284–5290, 1998.
15. Jones, B. E., Lo, C. R., Liu, H., Srinivasan, A., Streetz, K., Valentino, K. L., and Czaja, M. J. Hepatocytes sensitized to tumor necrosis factor-α cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. J. Biol. Chem., *275:* 705–712, 2000.
16. Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J. C., and Perucho, M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science (Wash. DC), *275:* 967–969, 1997.
17. Cory, S., and Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer, *2:* 647–656, 2002.

18. Newmeyer, D. D., and Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell, *112:* 481–490, 2003.
19. Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell, *6:* 1389–1399, 2000.
20. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science (Wash. DC), *292:* 727–730, 2001.
21. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. Role of BAX in the apoptotic response to anticancer agents. Science (Wash. DC), *290:* 989–992, 2000.
22. Ionov, Y., Yamamoto, H., Krajewski, S., Reed, J. C., and Perucho, M. Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. Proc. Natl. Acad. Sci. USA, *97:* 10872–10877, 2000.
23. Carroll, A. G., Voeller, H. J., Sugars, L., and Gelmann, E. P. p53 oncogene mutations in three human prostate cancer cell lines. Prostate, *23:* 123–134, 1993.
24. Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat. Med., *5:* 280–285, 1999.
25. Abreu-Martin, M. T., Chari, A., Palladino, A. A., Craft, N. A., and Sawyers, C. L. Mitogen-activated protein kinase kinase kinase 1 activates androgen receptor-dependent transcription and apoptosis in prostate cancer. Mol. Cell. Biol., *19:* 5143–5154, 1999.
26. Fu, M., Wang, C., Wang, J., Zhang, X., Sakamaki, T., Yeung, Y. G., Chang, C., Hopp, T., Fuqua, S. A., Jaffray, E., Hay, R. T., Palvimo, J. J., Janne, O. A., and Pestell, R. G. Androgen receptor acetylation governs trans activation and MEKK1-induced apoptosis without affecting *in vitro* sumoylation and *trans*-repression function. Mol. Cell. Biol., *22:* 3373–3388, 2002.
27. Clarke, A., Maandag, E., van Roon, M., van der Lugt, N., van der Valk, M., Hooper, M., Berns, A., and te Riele, H. Requirement for a functional *Rb-1* gene in murine development. Nature (Lond.), *359:* 328–330, 1992.
28. Jacks, T., Fazeli, A., Schmitt, E., Bronson, R., Goodell, M., and Weinberg, R. Effects of an Rb mutation in the mouse. Nature (Lond.), *359:* 295–300, 1992.
29. Lee, E. Y-H. P., Chang, C-Y., Hu, N., Wang, Y-C. J., Lai, C-C., Herrup, K., Lee, W-H., and Bradley, A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature (Lond.), *359:* 288–294, 1992.
30. Wu, L., de Bruin, A., Saavedra, H. I., Starovic, M., Trimboli, A., Yang, Y., Opavska, J., Wilson, P., Thompson, J. C., Ostrowski, M. C., Rosol, T. J., Woollett, L. A., Weinstein, M., Cross, J. C., Robinson, M. L., and Leone, G. Extra-embryonic function of Rb is essential for embryonic development and viability. Nature (Lond.), *421:* 942–947, 2003.
31. English, H. F., Santen, R. J., and Isaacs, J. T. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate, *11:* 229–342, 1987.
32. Day, M. L., Foster, R. G., Day, K. C., Zhao, X., Humphrey, P., Swanson, P., Postigo, A. A., Zhang, S. H., and Dean, D. C. Cell anchorage regulates apoptosis through the retinoblastoma tumor suppressor/E2F pathway. J. Biol. Chem., *272:* 8125–8128, 1997.
33. Wang, Y., Hayward, S. W., Donjacour, A. A., Young, P., Jacks, T., Sage, J., Dahiya, R., Cardiff, R. D., Day, M. L., and Cunha, G. R. Sex hormone-induced carcinogenesis in Rb-deficient prostate tissue. Cancer Res., *60:* 6008–6017, 2000.
34. Stanbrough, M., Leav, I., Kwan, P. W., Bubley, G. J., and Balk, S. P. Prostatic intraepithelial neoplasia in mice expressing an androgen receptor transgene in prostate epithelium. Proc. Natl. Acad. Sci. USA, *98:* 10823–10828, 2001.
35. Heisler, L. E., Evangelou, A., Lew, A. M., Trachtenberg, J., Elsholtz, H. P., and Brown, T. J. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol. Cell. Endocrinol., *126:* 59–73, 1997.
36. Yeh, S., Hu, Y. C., Rahman, M., Lin, H. K., Hsu, C. L., Ting, H. J., Kang, H. Y., and Chang, C. Increase of androgen-induced cell death and androgen receptor transactivation by BRCA1 in prostate cancer cells. Proc. Natl. Acad. Sci. USA, *97:* 11256–11261, 2000.
37. Zhao, X., Gschwend, J. E., Powell, C. T., Foster, R. G., Day, K. C., and Day, M. L. Retinoblastoma protein-dependent growth signal conflict and caspase activity are required for protein kinase C-signaled apoptosis of prostate epithelial cells. J. Biol. Chem., *272:* 22751–22757, 1997.
38. Bowen, C., Spiegel, S., and Gelmann, E. P. Radiation-induced apoptosis mediated by retinoblastoma protein. Cancer Res., *58:* 3275–3281, 1998.
39. Bowen, C., Birrer, M., and Gelmann, E. P. Retinoblastoma protein-mediated apoptosis after γ-irradiation. J. Biol. Chem., *277:* 44969–44979, 2002.
40. Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L., and Lin, B. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc. Natl. Acad. Sci. USA, *99:* 11890–11895, 2002.
41. Segawa, T., Nau, M. E., Xu, L. L., Chilukuri, R. N., Makarem, M., Zhang, W., Petrovics, G., Sesterhenn, I. A., McLeod, D. G., Moul, J. W., Vahey, M., and Srivastava, S. Androgen-induced expression of endoplasmic reticulum (ER) stress response genes in prostate cancer cells. Oncogene, *21:* 8749–8758, 2002.
42. Riegman, P. H., Vlietstra, R. J., van der Korput, J. A., Brinkmann, A. O., and Trapman, J. The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol. Endocrinol., *5:* 1921–1930, 1991.
43. Lu, S., Liu, M., Epner, D. E., Tsai, S. Y., and Tsai, M. J. Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. Mol. Endocrinol., *13:* 376–384, 1999.
44. Wang, L. G., Ossowski, L., and Ferrari, A. C. Overexpressed androgen receptor linked to p21WAF1 silencing may be responsible for androgen independence and resistance to apoptosis of a prostate cancer cell line. Cancer Res., *61:* 7544–7551, 2001.
45. Li, P., Lee, H., Guo, S., Unterman, T. G., Jenster, G., and Bai, W. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. Mol. Cell. Biol., *23:* 104–118, 2003.
46. Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell, *104:* 719–730, 2001.